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Comparative genomics reveals diversity among xanthomonads infecting tomato and pepper

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Abstract

Background: Bacterial spot of tomato and pepper is caused by four *Xanthomonas* species and is a major plant disease in warm humid climates. The four species are distinct from each other based on physiological and molecular characteristics. The genome sequence of strain 85-10, a member of one of the species, *Xanthomonas euvesicatoria (Xcv)* has been previously reported. To determine the relationship of the four species at the genome level and to investigate the molecular basis of their virulence and differing host ranges, draft genomic sequences of members of the other three species were determined and compared to strain 85-10.

Results: We sequenced the genomes of *X. vesicatoria* (*Xv*) strain 1111 (ATCC 35937), *X. perforans* (*Xp*) strain 91-118 and *X. gardneri* (*Xg*) strain 101 (ATCC 19865). The genomes were compared with each other and with the previously sequenced *Xcv* strain 85-10. In addition, the molecular features were predicted that may be required for pathogenicity including the type III secretion apparatus, type III effectors, other secretion systems, quorum sensing systems, adhesins, extracellular polysaccharide, and lipopolysaccharide determinants. Several novel type III effectors from *Xg* strain 101 and *Xv* strain 1111 genomes were computationally identified and their translocation was validated using a reporter gene assay. A homolog to Ax21, the elicitor of XA21-mediated resistance in rice, and a functional Ax21 sulfation systems were identified in *Xcv*. Genes encoding proteins with functions mediated by type II and type IV secretion systems have also been compared, including enzymes involved in cell wall deconstruction, as contributors to pathogenicity.

Conclusions: Comparative genomic analyses revealed considerable diversity among bacterial spot pathogens, providing new insights into differences and similarities that may explain the diverse nature of these strains. Genes specific to pepper pathogens, such as the O-antigen of the lipopolysaccharide cluster, and genes unique to individual strains, such as novel type III effectors and bacteriocin genes, have been identified providing new clues for our understanding of pathogen virulence, aggressiveness, and host preference. These analyses will aid in efforts towards breeding for broad and durable resistance in economically important tomato and pepper cultivars.

Background

Bacterial spot disease of tomato and pepper presents a serious agricultural problem worldwide, leading to significant crop losses especially in regions with warm and humid climate. The disease is characterized by necrotic lesions on leaves, sepals and fruits, reducing yield and fruit quality [1]. The disease is caused by a relatively diverse set of bacterial strains within the genus

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Xanthomonas; strain nomenclature and classification for the strains that infect pepper and tomato have gone through considerable taxonomic revision in recent years. Currently, the pathogens are classified into four distinct pathogen groups (A, B, C, and D) within the genus *Xanthomonas*. Strains belonging to groups A, B and D infect both tomato and pepper. Group C strains are pathogenic only on tomato [2,3]. These phenotypically and genotypically distinct strains have different geographic distributions. Strains of group A and B are found worldwide. C strains have been increasingly found in the U.S., Mexico, Brazil, Korea and regions bordering



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the Indian Ocean, and D group strains are found in the former Yugoslavia, Canada, Costa Rica, U.S, Brazil and regions of the Indian Ocean [4-8]. Three of the four groups except for D were originally described as a single pathovar within *Xanthomonas campestris* and referred to as *X. campestris* pv. *vesicatoria*. The D group consisted of a strain isolated from tomato that had been designated '*Pseudomonas gardneri*' for many years [9] although De Ley provided evidence for placement in the genus *Xanthomonas* [10]. Subsequently all four groups were classified as separate species on the basis of physiological and molecular characteristics as follows: *Xanthomonas euvesicatoria* (group A), *Xanthomonas vesicatoria* (group B), *Xanthomonas perforans* (group C), and *Xanthomonas gardneri* (group D) [11].

Based on 16S rRNA analysis, X. euvesicatoria strain 85-10 (A group) and X. perforans (C group) together form a monophyletic group, whereas X. vesicatoria (B group) and X. gardneri (D group) cluster together with X. campestris pv. campestris (Xcc) Xcc strain 33913 [11]. Recently, a phylogenetic tree was constructed based on MLST (multi-locus sequence typing) data for A, B, C and D group strains and other xanthomonads [12]. The MLST approach revealed that X. euvesicatoria and X. perforans form a group along with X. citri strain 306 (Xac). X. gardneri is most closely related to X. campestris pv. campestris strains while X. vesicatoria forms a distinct clade [12]. This diversity among the four groups makes the Xanthomonas-tomato/pepper system an excellent example to study pathogen co-evolution, as distinct species have converged on a common host.

While integrated management approaches for control of bacterial spot disease are available, the development of host resistance is more economical and environmentally benign for the control of the disease [13,14]. Host resistance may also be required to replace the loss of some integrated management tools. Use of copper and streptomycin sprays over the years, for example, has led to the development of resistant strains [5]. At the same time, genetic resistance has been lost due to race shifts in pathogen populations [15-17]. Designing new and possibly durable resistance requires knowledge of pathogenicity factors possessed by the four groups.

Many candidate pathogenicity factors have been identified in strains of *Xanthomonas*. A number of virulence factors are employed by xanthomonads to gain entry into leaf or fruit tissue, and gain access to nutrients, while simultaneously overcoming or suppressing plant defenses. Different secretion systems and their effectors have been shown to contribute to the virulence of plant pathogens. The type III secretion system (T3SS) encoded by the *hrp* (Hypersensitive Response and Pathogenicity) gene cluster [18,19] and type III secreted effectors have been widely studied for their role in hypersensitivity and pathogenicity. Effectors common between strains are believed to be responsible for conserved virulence function and avoidance of host defense. Differences in effector suites have evolved in closely related strains of plant pathogens and strain-specific effectors may help to escape recognition by host-specific defenses [20-25]. Important insights into pathogenicity mechanisms of X. euvesicatoria strain 85-10 (hereafter, *Xcv*) have been obtained with its genome sequence [26]. Here we report draft genome sequences of type strains of the other three bacterial spot pathogen species: X. vesicatoria strain 1111 (Xv 1111) (ATCC 35937), X. perforans strain 91-118 (Xp 91-118), and X. gardneri strain 101 (Xg 101) (ATCC 19865). We have annotated and analyzed predicted pathogenicity factors in the draft genomes. Additionally, we have investigated differentiation between xanthomonads that might explain differences in disease phenotypes and in host range.

Results and Discussion

Draft genome sequences of Xv strain 1111, Xp strain 91-118 and Xg strain 101 were obtained by combining Roche-454 (pyrosequencing) and Illumina GA2 (Solexa) sequencing data

Initially, we sequenced Xv strain 1111 (ATCC 35937) (hereafter Xv), Xp strain 91-118 (hereafter Xp) and Xgstrain 101 (ATCC 19865) (hereafter Xg) by 454 pyrosequencing [27]. De novo assembly using Newbler assembler resulted in 4181, 2360 and 4540 contigs, respectively, for Xv, Xp and Xg, with approximately 10fold coverage for each strain (Additional file 1: Table S1). Many pathogenicity genes, including type III effectors, existed in the form of fragments given the relatively low coverage of the 454-based assembly. More complete assemblies were obtained using Illumina sequencing [28]. De novo assemblies of around 100-fold coverage were constructed from the Illumina data alone or combined with pre-assembled 454 long reads using CLC Genomic Workbench [29]. Combined 454 and Illumina sequencing produced a much better assembly than either technology alone (Table 1). Therefore, combined assemblies were chosen for all subsequent analyses. The average contig size in the combined 454 and Illumina assemblies was around 18 kb for Xv and Xp, and 10 kb for Xg. The N50 (minimum number of contigs needed to cover 50% of the assembly) values were 37 and 40 for Xv and Xp, respectively, and 83 for Xg indicating that final assemblies consist of a few large contigs allowing reasonably accurate whole genome comparisons.

The three strains were deduced to contain plasmids as evidenced by the presence of genes that are known to be involved in plasmid maintenance (e.g. *parB/F* genes). We have used adjacency to such genes to infer occurrence of certain other genes on plasmids.

	Xanthomonas vesicatoria (Xv)	Xanthomonas perforans (Xp)	Xanthomonas gardneri (Xg)
Number of contigs	296	291	552
N50*	37	40	83
Mean contig length	18,686	18,082	10,014
Longest contig	153,834	133,836	88,536
Total length of contigs	5,531,090	5,262,127	5,528,125

Table 1 General sequencing and combined (454 and solexa) *de novo* assembly features of draft genomes of *Xv*, *Xp* and *Xg*

*N50 - number of contigs that cover 50% of the genome assembly.

Relationships of the strains to other xanthomonads using whole genome comparisons

16S rRNA analysis and MLST-based phylogenetic analysis showed the diversity among the four bacterial spot species. We carried out phylogenetic analysis based on orthologous protein-coding genes from draft genomes and reference xanthomonads (Figure 1). Whole genome comparisons were performed using the MUMi index [30] to assess pairwise distance between the draft genomes and available reference Xanthomonas genomes as shown in the phylogenetic tree and the distance matrix (Additional file 2: Fig. S2). Another program, dnadiff, based on nucmer [31] showed the extent of homologies among the shared regions of the genomes by pairwise comparisons (Additional file 3: Table S3). All of the methods yielded consistent results: we were able to ascertain that among the three newly sequenced strains in relationship to the previously sequenced strains, Xp and Xcv form the closest pair, which is in turn closest to Xac. Next, Xg is closest to Xcc, with Xv forming a clade with Xg and the Xcc species group (Figure 1, Additional files S2 and S3).

Four xanthomonads show variation in the organization of the type III secretion gene clusters

Annotation of the respective type III secretion gene clusters, or *hrp* genes showed that *Xp* has an almost identical and syntenic *hrp* cluster to that of *Xcv* (Figure 2). The most notable difference is that *hpaG* and *hpaF* encode the fusion protein XopAE in *Xp*, while they are present as separate genes in Xcv. Adjacent hypothetical protein XCV0410 (126 amino acid protein) is absent from *Xp*. *Xv* and Xg show greater similarity to the core hrp cluster genes of *Xcc* than to that of *Xcv*. *Xv* and *Xg* contain hrpW associated with the hrp cluster as in Xcc. Additionally, *xopD* in Xv and Xg is not associated with the *hrp* cluster as in Xcc (referred to as psv in Xcc). PsvA shows 74% and 84% sequence identity to the respective homologs from Xv and Xg. XopA (hpa1) from Xcv seems to be absent from Xv and Xg. Interestingly, we found a novel candidate effector gene (named xopZ2) upstream of *hrpW* in *Xv* and *Xg* (See below, Additional file 4: Fig. S4).



Concatenated amino acid sequences of the orthologous genes from four bacterial spot pathogen strains along with other sequenced xanthomonads were considered in the analysis. *Stenotrophomonas maltophilia* (*Sm*) was used as an outgroup. The evolutionary history was inferred using the Maximum likelihood method. The tree is drawn to scale, with branch lengths corresponding to the evolutionary distances. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.



Finally, the *hrp*-associated effector *xopF1* is conserved and intact in all four tomato and pepper pathogens.

A reporter gene assay confirms translocation of novel type III effectors

We identified and annotated T3SS effectors from the three newly sequenced xanthomonads (See Methods). Several candidate effectors, which had not yet been experimentally confirmed in xanthomonads, and candidate effectors with plausible translocation motifs were identified (Tables 2, 3, and 4). Corroborative evidence for T3SS-mediated translocation of the candidate effectors was assessed by constructing fusion genes with the C-terminal end of AvrBs2 coding sequence (avrBs2_{62-574aa}) in a race 6 strain of *X. euvesicatoria*. Translocation was measured in pepper cv. ECW 20R, containing the resistance gene *Bs2* (Additional file 4: Fig. S4). Genes *xopAO*, *xopG*, *xopAM*, and XGA_0724

(belonging to the *avrBs1* class of effectors), of which homologs were previously found in *Pseudomonas* species, were demonstrated to direct AvrBs2-specific hypersensitive reactions in ECW 20R (Tables 3, Table 4, Additional file 4: Fig. S4). Another candidate effector gene *xopZ2*, associated with the *hrp* clusters in Xv and Xg (Figure 2), was also functional in the AvrBs2-based assay. Thus, we identified five effectors (*xopAO*, *xopG*, *xopAM*, *xopZ2*, XGA_0724) that have not been previously recognized in *Xanthomonas* and showed their functionality.

Core effectors among four xanthomonads give insight into infection strategies of the pathogen

Comparing the draft genome sequences of the three xanthomonads with that of Xcv allowed us to identify the core effectors conserved in all four strains as well as strain-specific effectors (Tables 2, 3, and 4).

Effector class	Хсv	Xv	Хр	Хд	Pfam domains	References
AvrBs2	XCV0052	XVE_4395	XPE_2126	XGA_3805	Glycerophosphoryl diester phosphodiesterase	[104]
XopD	XCV0437	XVE_2372	XPE_2945	XGA_3151	C48-family SUMO cysteine protease (Ulp1 protease family); EAR motif	[105]
XopF1	XCV0414	XVE_3220	XPE_2922	XGA_2763	-	[105]
ХорК	XCV3215	XVE_0354	XPE_1077	XGA_3563	-	[106]
XopL	XCV3220	XVE_0359	XPE_1073	XGA_0320	LRR protein	[107]
ХорN	XCV2944	XVE_0564	XPE_1640	XGA_0350	ARM/HEAT repeat	[108]
XopQ	XCV4438	-	XPE_0810	XGA_0949	Inosine uridine nucleoside N-ribohydrolase	[105]
XopR	XCV0285	XVE_0593	XPE_1215, XPE_3295	XGA_1761	-	[106]
ХорХ	XCV0572	XVE_ 3610 XVE_3609 (partial)	XPE_1488 XPE_1553	XGA_3272 (second copy with frameshift)	-	[109]
XopZ1	XCV2059	+ (*)	XPE_2869	+(*)	-	[106]
XopAD	XCV4315/ 4314/4313	XVE_4177	XPE_4269	XGA_0755	SKWP repeat protein	[110]

Table 2 Core effectors present in all four tomato and pepper xanthomonads

*Xv and Xg contain effector xopZ2 belonging to the same family xopZ.

Table 3 Type III effectors specific to each species

Effector	Locus tags	Effector homolog	Pfam domains/biochemical motifs	Comments/Reference
Effectors specific to	o Xv			
ХорЈ2	XVE_4840 (partial); XVE_3769 (partial)	AvrBsT	C55-family cysteine protease or Ser/Thr acetyltransferase	[40]
XopAG	XVE_2415	AvrGf1	-	[39]
ХорАІ	XVE_4756	XAC3230	-	[25]
Effectors specific to	o <i>Xg</i>			
class avrBs1	XGA_0724	AvrA (84% identity)	-	This study
AvrHah1 (Fragmented in assembly)	XGA_4845/XGA_3187	AvrBs3	Transcriptional activator, nuclear localization	AvrBs3 present in few euvesicatoria strains [41].
ХорАО	XGA_1250	AvrRpm1 (61% identity)	-	This study
XopAQ	XGA_2091	Rip6/rip11	No known domains	[46]
XopAS	XGA_0764/0765	HopAS1	No known domains	This study
Effectors specific to	o <i>Xp</i>			
XopC2	XPE_3585	Rsp1239	Haloacid dehalogenase-like hydrolase	[24]
ХорЈ4	XPE_1427	AvrXv4	SUMO protease (experimental); YopJ-like serine threonine acetyl transferase domain (predicted)	[38,105]
XopAF	XPE_4185	AvrXv3	Transcriptional activator domain	[37]
XopAE	XPE_2919	HpaF/G	LRR protein	[24]
Effectors specific to	o Xcv			
AvrBs1	XCVd0104	AvrBs1	-	[26]
XopC1	XCV2435	ХорС	Phosphoribosyl transferase domain and haloacid dehalogenase-like hydrolase	[105]
ХорЈ1	XCV2156	ХорЈ	C55-family cysteine protease or Ser/Thr acetyltransferase	[105]
ХорЈЗ	XCV0471	AvrRxv	C55-family cysteine protease or Ser/Thr acetyltransferase	[26]
ХорО	XCV1055		Unknown	[26]
ХорАА	XCV3785	ECF	Early chlorosis factor, proteasome/cyclosome repeat	[26]
ХорАІ	XCV4428	AvrRxo1	-	[26]

Effector class	Locus tags	Pfam domains	Comments/References
Effectors	common to all pepper	pathogens Xv, Xcv and Xg	
XopE2	XCV2280, XVE_1190, XGA_2887	Putative transglutaminase	[114]
XopG	XCV1298, XVE_4501, XGA_4777	M27 family peptidase clostridium toxin	This study
Effectors	common to <i>Xv, Xg</i> but	absent from Xp and Xcv	
ХорАМ	XVE_4676, XGA_3942	-	This study
HrpW	XVE_3222, XGA_2761	Pectate lyase	HrpW associated with hrp cluster, May not be T3SE [111]
AvrXccA1	XVE_5046, XGA_0679	LbH domain containing hexapeptide repeats (X-[STAV]-X- [LIV]-[GAED]-X)- acyltransferase enzyme activity	May not be T3SE [112]
XopZ2	XGA_2762, XVE_3221	Not known	This study; Associated with hrp cluster.
Effectors	common to Xg and Xc	v but absent from Xp and Xv	
ХорВ	XGA_4392, XCV0581	-	[113]
Effectors	common to Xp and Xc	v but absent from Xg and Xv	
XopE1	XPE_1224, XCV0294	Putative transglutaminase	[114]
XopF2	XPE_1639, XCV2942	-	[105]
Xopl	XPE_3711, XCV0806	F-box domain	[115]
ХорР	XPE_3586, XPE_4695 (Partial), XCV1236		[105]
XopV	XPE_4158, XCV0657	-	[106]
ХорАК	XPE_4569, XCV3786	-	Not confirmed to be effector in Xanthomonas; Homolog of effector in <i>Pseudomonas</i> .
ХорАР	XPE_1567, XCV3138	Lipase class III	45% identity to homolog in <i>Xp</i> ; Homolog of rip38 from R. solanacearum RS1000 [46]
Effectors	present in Xv and Xp b	ut absent from Xg and Xcv	
XopAR	XVE_3216, XPE_2975	-	[46]

Table 4	Effectors	specific t	to	particular	groups	of	species
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At least 11 effector genes form a core set of common effectors for xanthomonads infecting tomato and pepper (Table 2). Of these 11, eight effector genes (avrBs2, xopK, xopL, xopN, xopQ, xopR, xopX and xopZ) were found to be conserved in all sequenced xanthomonads including the three draft genomes presented here with the exceptions of X. albilineans and X. campestris pv. armoraciae. These genes might be necessary for maintaining pathogenicity of these xanthomonads in a wide range of host plants. XopN has been reported to suppress PAMP (pathogen-associated molecular pattern)triggered immunity by interacting with tomato TARK1 and TFT1 [32]. XopF1 is conserved in tomato and pepper xanthomonads. Although a homolog of *xopF1* is found in *Xcc*, the respective gene is truncated [34]. Hence, *xopF1* is a potential pathogenicity determinant in tomato. A *xopF1* deletion mutant of *Xcv* did not show any difference in virulence when compared to wild type Xcv on the susceptible cultivar of pepper ECW, suggesting XopF1 is not the lone factor for pathogenicity of Xcv on pepper [33]. Another effector gene, xopD, is associated with the *hrp* gene cluster in *Xcv* and *Xp*. However, *xopD* appears to have translocated to another location in the genome in case of Xg, Xv and Xccstrains. XopD is annotated as "Psv virulence protein" in Xcc genome [34] and has been shown to be a chimeric protein sharing a C terminus with XopD from Xcv [35]. Although *xopD* homologs from Xv and Xg are syntenic with the psv gene in Xcc, Xv and Xg have intact fulllength copies of *xopD* as in *Xcv*, indicating that the xopD could be another effector exclusive to the tomato pathogens and a possible pathogenicity determinant in tomato. XopD has been shown to enhance pathogen survival in tomato leaves by delaying symptom development [36]. Two tandem copies of xopX are found in Xg. However, one gene in Xg appears to be inactive due to a frameshift mutation. In Xp, the two copies of xopX are found in different locations in the genome with neighboring genes, including chaperone gene groEL, which is also duplicated. Orthologs of xopZ are also found in all four xanthomonads, with 82% identity for Xcv and Xpand 35% identity for Xg and Xv. Apart from low sequence identity in Xv and Xg, gene-specific rearrangements appear to have occurred within each ortholog. We propose that the overall low amino acid relatedness (pairwise sequence identities below 50%) of this effector

in Xv and Xg warrants assigning the proteins to a new family within the *xopZ* class, named *xopZ2*, while the orthologs from *Xcv* and *Xp* belong to family of *xopZ1* as originally described in *Xoo* and as supported by pairwise sequence identities of at least 60% (see above, Figure 2, Table 4).

Effectors unique to Xp might be responsible for restricting growth on pepper

Xp is pathogenic only on tomato. The avirulence gene, *avrXv3*, present in *Xp*, was previously shown to elicit an hypersensitive response (HR) in pepper cv. ECW [37]. An *avrXv3* knockout mutant of *Xp* is not virulent in pepper cv. ECW indicating that other factors are associated with host specificity. Comparing effector repertoires of the pepper pathogens *Xg*, *Xcv*, and *Xv* with *Xp* may provide clues to the factors that are responsible for reduced virulence (Table 4). Besides avrXv3, the only effectors present in Xp and absent or inactive in Xg, Xvand Xcv are xopC2, xopAE and xopJ4 (avrXv4) (Table 3). The gene *avrXv4* is absent from other sequenced xanthomonads and shows gene-for-gene interaction with the Xv4 resistance gene from the wild tomato relative Solanum pennellii but does not contribute to restricted growth of Xp on pepper [38]. The effector xopC2 is a homolog of the effector rsp1239 from Ralstonia solanacearum GMI1000 and xopAE encodes an LRR protein with homology to the R. solanacearum effector PopC. Both genes, *xopC2* and *xopAE*, are truncated in Xcv. Therefore, these two effectors may trigger immunity in pepper. Interestingly, Xp contains a paralog of *xopP*. The two copies are found next to each other in the genome and share 75% identity at the amino acid level. The second copy is next to the candidate effector *xopC2*, which is unique to *Xp* among tomato and pepper pathogens. Effectors *xopC2* and *xopP* may both act to restrict growth in pepper. Moreover, there are at least two effectors, *xopE2* and *xopG*, present in the pepper pathogens Xcv, Xv and Xg but absent from Xp. These effectors may be essential pathogenicity factors in pepper.

Species-specific effectors

 $X\nu$ possesses two unique effector genes, xopAG (avrGf1) and xopAI (Table 3). A phylogenetic analysis of xopAGshowed that xopAG from $X\nu$ is closely related to xopAGfrom X. citri A^w , which has been shown to be responsible for causing an HR on grapefruit [39]. XopAI is a chimeric protein, which contains a conserved myristoylation motif at its N terminus, like XopJ1. This effector class also includes the homolog XAC3230 from Xac as well as XAUB_26830 and XAUC_23780 from X. fuscans subsp. aurantifolii strains B and C, respectively [25]. The presence of transposons and phage elements in close proximity helps to explain the evolution of this novel effector in *Xac* by terminal reassortment [35]. *Xv* also contains effector gene *avrBsT*, which is responsible for the hypersensitive response on pepper. Loss of the plasmid containing *avrBsT* in *Xcv* strain 75-3 allows the strain to cause disease on pepper [40].

Xg contains at least two effectors, avrHah1 (an avrBs3-like effector gene) and xopB as does Xcv, and share sequence identity of 82% and 86% respectively to the corresponding effectors of Xcv. However, AvrHah1 appears to specify a different phenotype when compared to avrBs3 from Xcv. AvrHah1 was shown to be responsible for increased watersoaking on pepper ECW-50R and 60R, whereas Xcv strains carrying avrBs3 show a phenotype that consists of small raised fleck lesions on pepper [41]. Another effector gene, *xopB*, has a PIP box at the 5' end in *Xcv*, whereas the homolog in *Xg* does not contain a PIP box. Neighboring genes to xopB in the respective strains are completely different between genomes, suggesting lack of synteny between the two species in this region (Table 4). XopB from Xg is 92% identical at the amino acid level to the homolog in Xcv. Deletion mutants of *xopB* from *Xcv* did not show any difference in virulence, indicating it does not contribute significantly to virulence [42]. However, *xopB* may contribute to virulence in Xg. We also identified eight effector genes that are unique to *Xcv* (Table 3). With the exception of xopAA (early chlorosis factor), all of these genes belong to regions of low GC content compared to average genome GC content (64.75%): avrBs1 (42%), xopC1 (48%), xopJ1 (xopJ) (57%), xopJ3 (avrRxv) (52%), xopO (52%), xopAJ (avrRxo1) (51%).

Few effectors are shared among phylogenetically related group strains

Although Xp and Xcv, and Xv and Xg form distinct phylogenetic groups (Figure 1), relatively few effectors are shared between these species. For Xp and Xcv, they share at least six effectors - xopE1, xopF2, xopP, xopV, xopAK, xopAP, which are absent from the other two genomes (Table 4). Xv and Xg appear to be most closely related to strains of X. *campestris* pv. *campestris*, and this relationship is reflected in the suite of effector genes. In fact, Xg and Xv share four effector genes with Xcc, namely, xopAM, avrXccA1, hrpW and xopZ2, with the caveat that hrpW and avrXccA1 may not function as intracellular effectors (Table 4). Furthermore, the genomic regions containing these genes are syntenic in Xg, Xv and Xcc.

X. gardneri shows evidence of effector acquisition by horizontal gene transfer

Effector homologs of *avrA*, *hopAS1* and *avrRpm1* from *P. syringae* pv. *tomato* T1 and *P. syringae* pv. *syringae*

B728a are found in Xg with 79%, 41% and 61% identity at the amino acid level, respectively (Table 3, Additional file 4: Fig. S4). Other X. gardneri strains also contain these effectors based on PCR screening (data not shown). These three effectors, XGA_0724 (belonging to avrBs1 class), XGA_0764/XGA_0765 (xopAS) and XGA_1250 (xopAO), are unique to X. gardneri. The C terminal region of XGA 0724 shows 53% identity to avrBs1 from Xcv. Hence according to the Xanthomonas effector nomenclature [24], XGA_0724 from Xg was placed under the class avrBs1. XGA_0764/XGA_0765 and XGA_1250 have not yet been reported to be found in xanthomonads and were assigned to new classes xopAS and xopAO. X. gardneri strains have been found to be associated with tomato and have a lower optimum temperature for disease development similar to that of pathovars of *Pseudomonas syringae* [43]. A high score by Alien_hunter analysis [44], along with very low GC content (45% for XGA_0724 and 48% for XGA_01250, 59% for XGA_0764/XGA_0765) and the proximity of mobile genetic elements provides evidence for horizontal gene transfer (Additional file 5: Table S5). Effector xopAS appears to be separated into two ORFs XGA_0764 and XGA_0765 by internal stop codon. The functionality of effector *xopAS* needs to be confirmed by in planta reporter gene assay. AvrA of P. syringae pv. tomato PT23 was shown to contribute to virulence on tomato plants [45]. Acquisition of XGA_0724 by Xg might have conferred increased virulence on tomato. AvrRpm1 from P. syringae pv. syringae possesses a myristoylation motif, which is absent from homologs in Xg. This modification in Xg might have been acquired to escape host recognition. Another candidate effector gene, xopAQ, in Xg is found 68 bps downstream of a perfect PIP box. The gene shows 65% identity at the amino acid level to rip6/11, a novel effector from R. solanacearum RS1000 [46].

All four xanthomonads contain Ax21 coding gene but only *Xcv* contains a functional sulfation gene

The *ax21* (activator of XA21-mediated immunity) gene is conserved among *Xanthomonas* species and is predicted to encode a type I-secreted protein that may serve as a quorum sensing signaling molecule [47]. A 17-amino acid sulfated peptide from the N-terminal region of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) Ax21 (axY^S22) was shown to bind and activate the XA21 receptor kinase from rice, demonstrating that Ax21 is a conserved PAMP that can activate plant immune signaling [48]. The *ax21* gene is present in *Xcv* (93% identity with *Xoo* PXO99 protein), *Xp* (94%), *Xv* (91%), and *Xg* (88%). The axY^S22 peptide is 100% conserved in *Xcv*, *Xp* and *Xv*, while in *Xg* there is a change from leucine to isoleucine at residue 20; this is unlikely to alter the activity of the peptide, since changing this residue to alanine had no effect on recognition by XA21 [48].

Recognition of axY^S22 by the XA21 receptor requires sulfation of tyrosine 22, which requires the putative sulfotransferase RaxST. In contrast to ax21, the raxSTgene is more variable in these genomes, which is consistent with a report of sequence differences in this gene among *Xoo* strains [49]. Furthermore, in *Xp*, there is a single-nucleotide insertion at position 65, causing a frameshift mutation. The *Xv* and *Xg* genomes do not contain *raxST*; therefore, the *ax21* gene products may be nonfunctional in these strains. These findings have implications for the further study of the role of Ax21 in quorum sensing and virulence, as well as for the usefulness of the XA21 receptor to confer resistance to xanthomonads in crop plants.

Two type II secretion systems are conserved in all four *Xanthomonas* genomes

Most cell-wall degrading enzymes, such as cellulases, polygalacturonases, xylanases, and proteases, are secreted by a type II secretion system (T2SS). The Xps T2SS, present in all xanthomonads, has been studied for its contribution to virulence in *Xcc* and *Xoo* [50,51]. Another T2SS cluster, known as the Xcs system, is found only in certain species of *Xanthomonas*, e.g. *Xcc*, *Xac*, and *Xcv*. The Xps system secretes xylanases and proteases and is under control of *hrpG* and *hrpX* [52], indicating differential regulation. Both Xps and Xcs systems are present in all three draft genomes.

Xanthomonads possess diverse repertoires of cell-wall degrading enzymes, which are present in diverse genomic arrangement patterns

Each species of *Xanthomonas* has its own collection of genes encoding endoxylanases, endoglucanases, and pectate lyases which contribute to cell wall deconstruction during pathogenesis. We have compared these repertoires from the three draft genomes and other xanthomonads as detailed in Table 5. The genes are designated for different families of glycosyl hydrolases (GH) and polysaccharide lyases (PL) that include the enzymes that cleave glycosidic bonds in the structural polysaccharides of plant cell walls.

Genes encoding secreted endoxylanases regulated by the *xps* genes have been described for their contributions to virulence, including XCV0965 [52] encoding GH30 endoxyalanase. The GH30 family catalyses the cleavage of methylglucuronoxylans in the cell walls of monocots and dicots at a β -1,4-xylosidic bond penultimate to one linking the xylose residue that is substituted by an α -1,2-linked 4-O-methylglucuronate residue [53,54]. Such an enzyme secreted by *Erwinia chrysanthemi* generates oligosaccharides that are not

Gene name	Family	Enzymatic function	Хр	Хас	Хсv	Xv	Хg	Xcc strain 33913	<i>Xoo</i> strain KACC
				Xylanases					
xyn10A	GH10	Endo-β-1,4-xylanase EC:3.2.1.8	2014	4254	4360	2337	1172	4118	4429
xyn10B			2016	4252	4358	-	-	-	4428
xyn10C			2020	4249	4355	2333	0341	4115	-
aguA	GH67	α-glucuronidase EC:3.2.1.139	4318	4227	4333	4712	2473	4102	4419
xyn51A	GH51	β-D-Arabino-furanosidase EC:3.2.1.55	0180	1286	1335	1029/1030	2303	1191	1317
xyn5A	GH5	Endo-β-1,4-xylanase EC:3.2.1.8	4682	0933/34 partial	0965	-	-	0857	3618
				Glucanases					
cel8A	GH8	Endo-1,4-β-D glucanase	1965	3516	3641	0432	-	-	-
cel9A	GH9		2345	2522	2704	1327	0588	2387	-
				Pectate lyases					
pel1A	PL1	Pectate lyase EC:4.2.2.2	3841	3562	3687	1933	4024	0645	0821
pel1B			1563	2986	3132	3512	0893	2815	-
pel1C			-	2373	2569	-	-	-	-
pel3A	PL3	Pectate lyase EC:4.2.2.2	-	2922	-	3222	2761	1219	-
pel4A	PL4	Rhamno-galacturonan lyase EC:4.2.2	1975	3505	3632	2592	4531	3377/78/79	1078
pel9A	PL9	Pectate lyase EC:4.2.2.2	-	-	2278	1927	1853	-	2265
pel10A	PL10	Pectate lyase EC:4.2.2.2	-	-	-	4069	5124	0122	-

Table 5 Repertoire of cell wall degrading enzymes in xanthomonads

Different cell wall degrading enzymes, such as xylanase, pectate lyase, glucanases, were compared for their repertoires among already sequenced xanthomonads including our draft genomes. Genes identified by their locus tags in the respective genomes.

assimilated for growth, suggesting a function in which it contributes to cell wall deconstruction for access to pectates for growth substrate [53]. It is interesting to note the orthologous genes encoding GH30 enzymes are absent in Xg and Xv, with a truncated xyn30 gene in Xac. On the basis of sequence homology, xyn30 genes may also contribute to virulence in Xoo, Xcc and Xp.

The more common GH10 endoxylanases, which occur in several bacterial and fungal phyla, have been implicated in the virulence of plant pathogenic bacteria and fungi [55,56]. In *Xoo*, deletion of the gene encoding a GH10 *xyn10B* resulted in diminished virulence [57]. All sequenced *Xanthomonas* genomes contain either two or three copies of *xyn10* genes, all of which are within a gene cluster that may comprise a single operon (Figure 3). The GH10 endoxylanases are the best studied of all of the xylanases, and structure/function relationships may be inferred on the basis of gene sequence. The action of these enzymes on glucuronoxylans generates xylotriose, xylobiose, and small amounts of xylose that generally serve as substrates for growth. Also generated is methylglucuronoxylotriose, that is formed to the extent that xylose residues in the β -1,4 xylan backbone are substituted with α -1,2-linked 4-O-methylglucuronate residues [58].

An adjacent gene cluster in an opposite orientation contains agu67 gene encoding a GH67 α -glucuronidase that serves to catayze the removal of 4-O-methylglucuronate from the reducing terminus of methylglucuronoxvlotriose. This activity provides a synergistic function to the overall xylanolytic process to generate xylotriose, which is converted to xylose by xylanases and xylosidases for complete metabolism [59]. The coregulation of operons encoding XynB and Agu67 enzymes occurs as a logical condition to coordinate expression of genes that encode these and additional enzymes that collectively process glucuronxylans and glucuronoarabinoxylans for complete metabolism. The accessory enzymes and transporters necessary for the function of these enzymes are embedded within these operons in Gram positive bacteria [60-62] and share similarities noted here with Xanthomonas spp.. These include the genes encoding two glycohydrolases, a β -xylosidase and an α -L-arabinofuranosidase. Also included in this cluster are genes encoding enzymes for intracellular metabolism of glucuronate and xylose, including glucuronate isomerase; xylulose isomerase; D-mannonate dehydratase; and D-mannonate oxidoreductase. Genes encoding mannitol dehydrogenase and the hexuronate transporter, as well as the TonB-dependent receptor and LacI transcriptional regulator, flank these two operons.

The arrangement and content of xylanolytic enzymes differentiate Xanthomonas species into three groups (Figure 3). Here, we propose a common nomenclature for xylanases, the genes for which have been annotated in the sequenced genomes. Members of the first group are Xac, Xcv and Xp in which all three genes encoding GH10 endoxylanases (xyn10A, xyn10B and xyn10C) are present, and with additional genes further downstream in this cluster. Members of the second group are *Xcc*, Xv and Xg in which genes encoding two of the three endoxylanases are present (xyn10A and xyn10C) and where one or more of the the downstream genes are absent. Xoo strains represent a third group in which a different set of two endoxylanase encoding genes are present (*xyn10A* and *xyn10B*)

and where the β -galactosidase and gluconolactonase genes flanking *xyn10C* are absent. It is noteworthy that the organization of genes in the cluster encoding the α -glucuronidase is conserved across Xanthomonas species.

Genes involved in several Type IV secretion systems are present in genomes and plasmids

Like Xcv, the tomato pathogens, Xg, Xv and Xp, also appear to contain more than one copy of a type IV secretion system (T4SS) cluster (Figure 4A, B). Two T4SS clusters (Vir and Dot/Icm type) are present in *Xcv*, and genes belonging to both of these systems are found on plasmids [26]. The Dot/Icm type system is absent from Xv, Xp and Xg.

In Xv and Xp, genes for one T4SS are on a plasmid and the second one on the chromosome while in Xg, two T4SS gene clusters are on a plasmid and one is on the chromosome. The two T4SS clusters on plasmids of Xg do not show any similarity to the genes for T4SS in Xac, Xcv, Xcc and Xoo. Of the two T4SS clusters in Xg,



containing three endoxylanase genes xyn10A, xyn10B and xyn10C; B) Found in Xcc, Xv and Xg containing two endoxylanases xyn10A and xyn10C; and C) Found in Xoo containing xyn10A and xyn10B within endoxylanase operon.



one is also found in $X\nu$ and Xp. This cluster appears to be exclusive to these three tomato pathogens (Figure 4A). The genes belonging to this cluster show low (30-45%) identity to the T4SS clusters from *Ralstonia, Burkholderia, Bradyrhizobium,* and *Stenotrophomonas maltophilia*. The other cluster from Xg, which is absent from $X\nu$ and Xp, shows very high identity (98%) and synteny to the T4SS cluster of *Burkholderia multivorans* and around 89% identity to a T4SS cluster of *Acidovorax avenae* subsp. *citrulli* (Figure 4B).

Apart from the plasmid borne T4SS genes, *Xcv* also contains a portion of a type IV system cluster on the chromosome and consists of *VirB6*, *VirB8*, *VirB9*, *VirD4* genes. This chromosomal cluster is flanked by a transposon element (IS1477) that might indicate its horizontal gene transfer. *Xp*, *Xg* and *Xv* genomes contain a complete chromosomal T4SS cluster showing high identity to the T4SS chromosomal clusters from *Xcc* (Figure 4C).

Type V secreted adhesins function in synergism during pathogenesis

Different adhesins have been shown to function at different stages of the infection process starting with attachment, entry, later survival inside host tissue and colonization by promoting virulence [63,64]. FhaB hemagglutinin, important for leaf attachment, survival inside plant tissue and biofilm formation, is present in all four tomato pathogens. In Xcv, fhaB is divided into two separate open reading frames, XCV1860 and XCV1861, with the two-partner secretion domains being present in XCV1860. Sequence alignment indicates that *fhaB* is possibly inactivated in Xcv by the internal stop codon that separates XCV1860 from XCV1861. In the case of Xoo PXO99A, the Xanthomonas adhesin-like proteins XadA and XadB promote virulence by enhancing colonization of the leaf surface and leaf entry through hydathode [64]. As in Xcv and Xac, Xp encodes

two copies of *xadA*, while *Xv* and *Xg* possess a single ortholog of *xadA* as does *Xcc*. YapH and the type IV pilus protein PilQ were shown to be involved in virulence in *Xoo* during later stages of growth and migration in xylem vessels. In *Xcv*, *Xc*, and *Xoo*KACC, two copies of *yapH* are present. There are two *pilQ* orthologs in *Xcv* and only one in other sequenced xanthomonads. Next to the *fhaB* and *fhaC* adhesin genes, *hms* operon is present in the genomes of xanthomonads, the homologs of which are *pga* operon genes in *E. coli* involved in bio-film formation [65].

Type VI secretion system is present in Xcv, Xv and Xp

Type VI secretion system (T6SS) has been shown recently to contribute to host pathogen interactions during pathogenesis in *Vibrio cholerae*, *Burkholderia pseudomallei* and *Pseudomonas aeruginosa*. Hcp (Haemolysin-coregulated protein) and Vgr (valine-glycine repeats) proteins are exported by the T6SS [66]. T6SS clusters can be assigned to three different types in xanthomonads (Table 6). *Xcv* and *Xp* possess two types of T6SSs (type 1 and 3); whereas *Xv* contains only a single type of T6SS, type 3. As in *Xcc*, there is no T6SS cluster in *Xg* (Table 6, Additional file 6: Table S6).

LPS locus displays remarkable variation in sequence and number of coding genes and shows host specific variation

The lipopolysaccharide (LPS) biosynthesis cluster has been studied in detail in *Xcc* [67], which comprises three regions; region 1 from *wxcA* to *wxcE* involved in

Table 6 Type VI secretion clusters in different xanthomonads

biosynthesis of water soluble LPS antigen; region 2 (gmd, rmd) coding for LPS core genes; and region 3 from wxcK to wxcO coding for enzymes for modification of nucleotide sugars and sugar translocation systems. This LPS biosynthesis locus is positioned between highly conserved housekeeping genes, namely cystathionine gamma lyase (metB) and electron transport flavoprotein (etfA), as reported in other xanthomonads [68]. Comparison of this cluster from draft genomes to the already sequenced xanthomonads revealed high variability in the number of genes and their sequences. Xv and Xg have an identical type of LPS gene cluster of 17.7 kb encoding 14 open reading frames (Figure 5A) which is similar in organization and sequence identity to the LPS locus from *Xcc* strains. Interestingly, *Xg* and *Xv* also contain two glycosyl transferases involved in synthesis of xylosylated polyrhamnan as seen in Xcc [69], in contrast to glycosyl transferases (wbdA1, wbdA2) involved in synthesis of polymannan in Xcv [26]. This suggests that basic structure of O-antigen in Xg and Xv is similar to Xcc. The three tomato/pepper pathogens Xcv, Xv and Xg have retained an ancestral type of LPS gene cluster (Figure 5A and 5B). On the other hand, Xp has acquired a novel LPS gene cluster during the course of evolution and is completely different in sequence and number of genes that are encoded. In *Xp*, this LPS locus is 17.3 kb long and encodes 12 ORFs, all of which are absent in the corresponding genomic region of Xcv, Xv or Xg. Also the first five ORFs flanking the *metB* side of the LPS locus in Xp (Figure 5A, ORFs colored in red) showed very low or no identity to region 1 of the LPS

Strain	T6SS #1	T6SS #2	T6SS #3
Phosphorylation-type regulators:	Kinase/Phosphatase/Forkhead	-	Kinase/Phosphatase/Forkhead
AraC-type regulators:	-	-	AraC
Xvv NCPPB702	YES	/	/
Xcm NCPPB4381	YES	/	/
Xaub	/	/	YES
Хаис	/	/	YES
Хас	/	/	XAC4116 - XAC4148
Xv	/	/	YES
Хр	YES	/	YES
Xcv	XCV2120 - XCV2143	/	XCV4206 - XCV4244
<i>Xoo</i> KACC10331	XOO3034 - XOO3052	XOO3466 - XOO3517	/
Xoo MAFF 311018	XOO2886 - XOO2906	XOO3286 - XOO3319	/
Xoo PXO99A	XOO0245 - XOO0270	XOO2029 - XOO2060	/
Xoc BLS256	XOC2523 - XOC2545	XOC1309 - XOC1370	/
Xg	/	/	/
Xcc ATCC33913	/	/	/
<i>Xcc</i> 8004	/	/	/
<i>Xca</i> 756C	/	/	/
Xalb	/	/	/



locus in the other xanthomonads. However, these ORFs still belong to the same Pfam families [70] that are usually present in this region, for example, ABC transporters and glycosyl transferases. The second half of the LPS cluster flanking *etfA* side encodes six ORFs, which are homologs of the LPS cluster genes from Xac, Xcm and Xoo. Phylogenetic insight based on conserved metB and *etfA* genes that flank the LPS locus suggest that the ancestor of all the Xanthomonas pathogens of pepper and tomato studied in this paper had the same LPS gene cluster, however putative horizontal gene transfer events at this locus have led to the acquisition of a novel LPS gene cluster in *Xp* (Figure 5B). Alien_hunter analysis also supports this acquisition with a high score showing this region to belong to an anomalous region (Additional file 5: Table S5). This event might have played a major role in changing the specificity of *Xp* towards tomato and its dominance over its relative(s) as reported previously [71], similar to variant epidemic strain of Vibrio cholerae, reported to be a major reason for its emergence and cholera outbreak during the 1990's in the Indian subcontinent [72]. Identity in terms of sequences and gene organization among pepper pathogens and absence of those genes from X. perforans and a novel LPS cluster in the tomato pathogen X. perforans suggest a role of this cluster in host specific variation.

Analysis of DSF cell-cell signaling system

RpfC/RpfG are two-component signaling factors and are involved in DSF (diffusible signal factor) cell-cell signaling [73-76], known to co-ordinate virulence and biofilm gene expression. The genomes of *Xv*, *Xp*, and *Xg* carry an *rpf* (regulation of *p*athogenicity *f*actors) gene cluster (Table 7) that is found in all xanthomonads and which encodes components governing the synthesis and perception of the signal molecule DSF [74,75]. The Rpf of the DSF system regulates the synthesis of virulence factors and biofilm formation and is required for the full virulence of Xcc, Xac, Xoc, and Xoo [77-81]. RpfF is responsible for the synthesis of DSF, whereas, RpfC and RpfG are implicated in DSF perception and signal transduction [73-76]. RpfC is a complex sensor kinase, whereas RpfG is a response regulator with a CheY-like receiver domain that is attached to an HD-GYP domain. HD-GYP domains act in degradation of the second messenger cyclic di-GMP [82]. In addition to genes encoding these products, *Xg* and *Xp* have *rpfH*, which encodes a membrane protein related to the sensory input domain RpfC but whose function is unknown. Xv contains rpfH but with an internal stop codon, whereas functional *rpfH* is present in *Xcv* and *Xcc*, and totally absent in *Xac* and *Xoo*.

Cyclic di-GMP signaling

Cyclic di-GMP is a second messenger known to regulate a range of functions in diverse bacteria, including the virulence of animal and plant pathogens [83-85].

Table 7 A comparison of rpf cluster from rpfB to rpfGfound across a range of Xanthomonas genomes

Gene Name	<i>Xcc</i> 8004	Хоо	Χсν	Xv	Хр	Хg
rpfB	XC_2331	XOO2868	XCV1921	2934	0530	2948
rpfF	XC_2332	XOO2869	XCV1920	2932	0528	2950
rpfC	XC_2333	XOO2870	XCV1919	2930	0526	2952
rpfH	XC_2334	Absent	XCV1918	2928/2926*	0524	2954
rpfG	XC_2335	XOO2871	XCV1917	2924	0522	2956

The cellular level of cyclic di-GMP is controlled by a balance between synthesis by GGDEF domain diguanylate cyclases and degradation by HD-GYP or EAL domain phosphodiesterases. GGDEF, EAL and HD-GYP domains are largely found in combination with other signaling domains, suggesting that their activities in cyclic di-GMP turnover can be modulated by environmental cues. A number of proteins involved in cyclic di-GMP signaling have been implicated in virulence of Xcc [86,87]. The genome of Xcv encodes 3 proteins with an HD-GYP domain and 33 proteins with GGDEF and/or EAL domains. As in other Xanthomonas spp., the HD-GYP domain proteins are completely conserved in Xcv, Xv, Xg and Xp. There is also almost complete conservation of GGDEF/EAL domain proteins between Xcv and three draft genomes, although $X\nu$ has no ortholog of XCV1982 (Additional file 7: Table S7). In addition, the EAL domain protein (XCVd0150) encoded on a plasmid in Xcv is absent in the other strains.

Copper resistance (*cop*) genes are present in Xv and copper homeostasis (*coh*) genes are present in all strains

Among the Xcv, Xv, Xp and Xg strains sequenced, Xv is the only one resistant to copper and the only strain harboring a set of plasmid borne genes, namely *copL*, copA, copB, copM, copG, copC, copD, and copF that are also present in copper resistant strains of Xac (unpublished data/Behlau, F. personal communication) and S. maltophilia [88]. Genes copA and copB have been previously annotated as copper resistance related genes for many different xanthomonad genomes including Xoo, Xoc, Xcv, Xac and Xcc. Homologs of these genes are also present in Xv, Xg and Xp and are located on the chromosome. Additionally, upstream of copA on the chromosome of all strains, there is an ORF that shares homology with plasmid copL. In contrast to what has been published, chromosomal copA and copB are not responsible for copper resistance but likely for copper homeostasis and/or tolerance. While strains harboring the plasmid-borne *cop* genes, like in Xv, are resistant to copper and can grow on MGY agar (manitol-glutamate yeast agar) amended with up to 400 mg L⁻¹ of copper sulfate pentahydrate, strains that have only the chromosomal *cop* genes as for *Xcv*, *Xp* and Xg, are sensitive to copper and can only grow on media amended up to 75 mg L⁻¹ of copper. Nucleotide sequence of plasmid *cop* genes in Xv are 98% similar to the ones found in Xac and Stenotrophomonas, whereas chromosomal copLAB from Xv is 83% identical to homolog ORFs in Xcv, Xg and Xp. When copL, copA and copB genes from Xv located on the plasmid are compared to the homologs on the chromosome of the same strain, the identity of nucleotide sequences is 27, 73, and 65%, respectively. To avoid further confusion or misinterpretation, we suggest that the nomenclature of the chromosomal *copL*, *copA* and *copB* genes in xanthomonads should be changed to *cohL*, *cohA* and *cohB*, respectively, referring to copper homeostasis genes. New nomenclature has been adopted in the annotation of the draft genomes.

Genes unique to *X. perforans* as compared to pepper pathogens give clues to its predominance over *Xcv* in the field and host specificity

Thirteen gene clusters were found to be specific to the tomato pathogen Xp when compared to the other three strains (Additional file 8: Table S8). A part of the clusters are syntenic to the genomic regions specific to the three pepper pathogens, suggesting the replacement of these genomic regions from pepper pathogens in correspond to these region in Xp. These replaced regions in Xp might provide potential candidates for host range determinants. Most notable among these regions was the LPS cluster genes (See above). Other such regions include the avirulence genes avrXv3 and avrXv4, a TIRlike domain containing protein, oxidoreductases, and bacteriocin-like proteins that were not found in any other sequenced xanthomonads. Importance of bacteriocin-like genes in Xp has already been studied for its predominance in the field over T1 strains [89,90]. Alien_hunter analysis showed that the bacteriocin BCN-A region belongs to an anomalous region indicating possible horizontal gene transfer of this region (Additional file 5: Table S5).

Pepper pathogenicity/aggressiveness factors increased *in planta* growth of *Xp*

Comparison of proteomes of Xv, Xg, Xcv against Xp showed 68 genes exclusive to pepper pathogens which might be candidate virulence factors on pepper (Additional file 9: Table S9). These include 16 genes with known function, 35 coding for mobile genetic elements, and 17 genes with unknown function/hypothetical proteins. Out of the 16 genes with known function, xopG was confirmed to be a type III effector using the avrBs2 reporter gene assay and 6 genes belong to the LPS biosynthesis gene cluster. These 16 genes were searched against already sequenced genomes of Xac, Xcc and Xoo. The *wxcO* gene, which codes for O-antigen, has been identified to be a virulence factor in the X. fuscans bean pathosystem by subtractive hybridization [91]. Three genes, XCV1298, XCV1839 and wxcO, were initially selected for the verification of their contribution to virulence in pepper. Individual genes along with their promoter regions were cloned into pLAFR3 and conjugated individually and in combination into X. perforans

ME24 (91-118 $\Delta avrXv3$), which no longer elicits an HR in pepper. However, *in planta* growth of ME24 is more similar to that of an avirulent strain than the virulent pepper strain TED3 race 6. ME24 transconjugants carrying *wxcO* and *XCV1839* in combination showed increased *in planta* growth and also comparatively increased number of lesions on pepper cv. ECW when compared to ME24 revealing that these two genes play in fact a role in pepper pathogenicity (Figure 6).

Genes specific to Xg as compared to other tomato/ pepper pathogens may explain its aggressive nature on tomato and pepper

Comparison of genes from *Xg* against *Xcv*, *Xp* and *Xv* genes showed the presence of 625 genes specific to Xg (Additional file 10: Table S10). These include four type III effectors (*avrBs1* member, *xopAO*, *avrHah1*, *xopAQ*), twenty-one genes belonging to the unique type IV secretion system cluster and associated genes. These genes can be speculated to contribute to the aggressive nature of Xg strains on tomato and pepper. Xg also contains a unique beta xylosidase not present in any other xanthomonads. Type II secreted beta xylosidase has been studied for its role in plant cell wall digestion. Moreover, Xg contains XGA_3730 coding for a hemolysin-type calcium-binding repeat containing protein, a homolog of which is found in *Xylella* strains with 55% sequence identity. In Xylella, this gene is annotated as a member of a family of pore forming toxins/RTX toxins. Its homolog is also found in other plant pathogens (i.e. P. syringae pv. syringae B728a and R. solanacearum GMI1000). This protein has been described as a type I effector in X. fastidiosa strain temecula (PD1506) [92]. RTX toxin family members, especially of the hemolysin type, have been shown to be virulence factors in a variety of cell types in eukaryotes [93,94]. Finally, a gene XGA_0603 coding for lanthionine



synthetase (lantibiotic biosynthesis) is found among these *Xg* specific genes, a homolog of which is found in *Xvm* NCPPB702. LanL enzymes in pathogenic bacteria contribute to virulence by modifying the host signaling pathways, in most cases by inactivating MAPKs [95].

Genes common to all tomato pathogens but absent from other sequenced xanthomonads

In order to see what defines the tomato pathogens, we compared the four sequenced genomes (Xv, Xp, Xg)and $Xc\nu$) to other sequenced xanthomonads. We found seven genes that were conserved in all four tomato pathogens and absent from most of other sequenced xanthomonads with the exception of Xcm, Xvv, Xaub and Xauc, which possess homologs for six out of these seven genes (Table 8). Only the hypothetical protein XCV2641 seems to be specific to the four tomato pathogens. This gene shows only 35% sequence identity to a gene from Xvv and Xcm. A homolog of the hypothetical protein, XCV4416 was found in Xau, but is absent from all other sequenced xanthomonads. Genes homologous in Xcm and Xvv include two transposase genes both belonging to the transposase 17 superfamily (XCV0615, XCV0623), XCV0041 (putative penicillin amidase fragment), XCV0111 (lignostilbenealpha, beta dioxygenase), XCV0112 (uncharacterized protein conserved in bacteria) (Table 8). Interestingly, XCV0111 encodes a protein known to be involved in phenylpropanoid degradation. Phenylpropanoids are well known plant secondary metabolites induced during defense response upon pathogen attack [96]. It appears that the four tomato pathogens along with Xvvand Xcm have acquired this function to disarm the basal plant defense.

The evolution of pathogenicity clusters corresponds to the MLST-based phylogeny

The correlation between tree topology using MLST and phylogeny based on the sequences of pathogenicity clusters and the *avrBs2* effector gene, which is found in all xanthomonads, was tested. Based on MLST, Xp and Xcv group together along with Xac while Xg is more closely related to Xcc. Xv forms a different clade and is more closely related to the Xcc group. As can be seen in Figure 7, phylogeny based on MLST is congruent with phylogeny based on the pathogenicity clusters (*gum*, *hrp* cluster) and based on the *avrBs2* effector, suggesting that overall these clusters were vertically inherited from the most recent common ancestor of these strains.

Conclusions

The interaction of *Xanthomonas* strains with tomato and pepper represents a model system for studying plantpathogen co-evolution because of the diversity present

Locus tag for <i>Xcv</i> 85-10	Possible function	Homolog present in any other genera	GC content
XCV0623	Transposase 17 superfamily Hypo protein -COG belonging to transposase, inactive derivatives	In Stenotrophomonas, Acidovorax Xanthomonas campestris pv. musacearum NCPPB4381	0.59
XCV2641	Hypothetical protein	X. c. musacearum and X. c. vasculorum (identity 37, 31% respectively)	0.65
XCV4416	Hypothetical protein	Pectobacterium carotovorum X. fuscans pv. aurantifolii	0.57
XCV0615	Transposase 17 superfamily Hypothetical protein COG1943 (transposase, inactivated derivates)	Acidovorax, X. c. musacearum and X. c. vasculorum	0.62
XCV0112	COG4704 uncharacterized protein conserved in bacteria	<i>Stenotrophomonas, X. c.</i> musacearum and <i>X. c.</i> vasculorum	0.65
XCV0111	putative lignostilbene-alpha,beta-dioxygenase-phenylpropanoid compound degradation	<i>Stenotrophomonas, Ralstonia, X. c.</i> musacearum and <i>X. c.</i> vasculorum	0.66
XCV0041	putative penicillin amidase (fragment)	<i>Ralstonia, X. c.</i> musacearum and <i>X. c.</i> vasculorum	0.64

Table 8 Genes present in all four tomato and pepper pathogens but absent from all other sequenced xanthomonads

among the strains causing bacterial spot. Although the four *Xanthomonas* species infect the same host, tomato, and cause very similar disease, they are genetically diverse pathogens. The comparative genomic analysis has provided insights into the evolution of these strains. Whole genome comparisons revealed that Xg and Xv are more

closely related to *Xcc* than *Xcv* and *Xp*. A few pathogenicity clusters, such as *hrp*, *xcs* and *xps* of *Xg* and *Xv*, were similar in terms of genetic organization and sequence identity to *Xcc* (Figure 8). However, a few pathogenicity clusters of the four strains belonging to four phylogenetic groups showed different evolutionary origins. While the





pepper pathogens Xcv, Xv and Xg possess similar LPS biosynthesis cluster, part of the LPS cluster from *Xp* is similar to the one from Xac (Figure 8). Xv contains few effectors, including *xopAG* (*avrGf1*) and *xopAI* the latter of which was previously found to be unique to citrus pathogens Xac, Xaub and Xauc [25]. Xg has a number of effectors homologous to P. syringae type III effectors suggesting probable horizontal transfer of these effectors. Xg contains a unique T4SS along with the one that is exclusive to Xp, Xv and Xg. Xp has two T6SSs, as found in Xcv. Xv has only one T6SS which is similar to that of *Xac. Xg* has no T6SS as seen for *Xcc* (Figure 8). While *Xg* and Xv show close relationship to Xcc based on whole genome comparisons, few pathogenicity clusters mentioned above seem to be conserved among tomato/pepper xanthomonads.

Type III effectors have been investigated for their contribution to pathogenicity and host-range specificity. In addition to homologs of the known effectors, we identified novel effectors in the draft genomes. By comparing effector repertoires of tomato pathogens, two possible candidate pathogenicity determinants, *xopF1* and *xopD*, were identified, of which *xopD* is responsible for delaying symptom development, and in turn, is important for pathogen survival. Unique genes present in Xg include the novel effectors xopAO, xopAQ, xopAS and an avrBs1 member as well as a few other virulence factors, which have been characterized in other plant pathogens and which could explain the aggressive nature of Xg on pepper. Each species contains at least three unique type III effectors, which could explain host preferences among the strains and their aggressiveness on tomato/pepper. Comparison of the LPS clusters between the four species revealed significant variation. Xp has acquired a novel LPS cluster during evolution, which might be responsible for its predominance and its limited host range. As seen from the *in planta* growth assay of Xp $\Delta avrXv3$ mutant carrying the LPS O-antigen from Xcv, the LPS cluster from pepper pathogens can be a contributor to the increased *in planta* growth of $Xp \ \Delta avrXv3$ mutant on pepper, but is not the absolute virulence determinant. Use of the XA21 receptor similar to the



Xoo-rice system in Xcv - tomato/pepper could be one of the ways to confer resistance to xanthomonads due to presence of a similar AX21 peptide and a functional rax system in Xcv. Common and unique genes encoding enzymes involved in cell wall deconstruction are candidates for further study to define host preference and virulence.

In conclusion, comparison of draft genomes obtained by next generation sequencing has allowed an in-depth study of diverse groups of bacterial spot pathogens at the genomic level. This analysis will serve as a basis to infer evolution of new virulent strains and overcoming existing host resistance. The knowledge of potential virulence or pathogenicity factors is expected to aid in devising effective control strategies and breeding for durable resistance in tomato and pepper cultivars.

Methods

Genome sequencing

Xv, Xp and Xg were sequenced by 454-pyrosequencing [27] at core DNA sequencing facility, ICBR, University of Florida. *Xanthomonas* isolates were grown overnight in nutrient broth. Genomic DNA was isolated using CTAB-NaCl extraction method [97] and resuspended in TE buffer (10 mM Tris pH 8, 1 mM EDTA pH 8). Libraries of fragmented genomics DNA were sequenced on 454-Genome Sequencer, FLX instrument at Interdisciplinary Center for Biotechnology Research (ICBR) at UF. *De novo* assemblies were constructed using 454 Newbler Assembler [27]. The three draft genomes were obtained with around 10× coverage.

For Illumina sequencing, the Xanthomonas strains were purified from single-colony and grown overnight in liquid cultures. Genomic DNA was isolated by phenol extraction and precipitated twice with isopropanol, and finally dissolved in TE buffer. DNA was then purified by cesium chloride density gradient centrifugation and precipitated with 95% ethanol, then dissolved in TE buffer. Libraries of fragmented genomic DNA with adapters for paired-end sequencing were prepared according to the protocol provided by Illumina, Inc. with minor modifications. The libraries were sequenced on the 2G Genome Analyzer at Center of Genome Research & Biocomputing at Oregon State University and post-processed using a standard Illumina pipeline [28]. We obtained approximately 8-10 million 60-bp reads for each genome, providing roughly 95× predicted coverage.

Assembly and annotation

De novo assembly was generated on Newbler assembler (version 2.3; 454 Life Science, Branford, CT) using 454sequencing reads for each genome. CLC workbench [29] was used in the next step for combining 454-based contigs with illumina reads, wherein, 454 based contigs were used as long reads to fill in gaps generated during combined *de novo* assembly. These combined assemblies of each genome were uploaded on IMG-JGI (Joint Genome Institute, Walnut Creek, California) server for gene calling. The gene prediction was carried out using Gene-Mark. Pfam, InterPro, COGs assignments were carried out for identified genes. Pathogenicity clusters described in the paper were manually annotated.

Whole genome comparisons

We aligned draft genomes against reference Xanthomonas genomes using nucmer [31] of MUMmer program (version 3.20) and dnadiff was used to calculate percentage of aligned sequences. We have also compared genomes using the MUM index [30] to measure distances between two genomes. The maximal unique exact matches index (MUMi) distance calculation was performed using the Mummer program (version 3.20). Mummer was run on concatenated contigs or replicons (achieved by inserting a string of 20 symbols 'N' between contig or replicon sequences) of each genome. The distance calculations performed using the MUMi script are based on the number of maximal unique matches of a given minimal length shared by two genomes being compared. MUMi values vary from 0 for identical genomes to 1 for very distant genomes [30].

Phylogenetic analysis

MLST sequences (fusA, gapA, gltA, gyrB, lacF, lepA) for all the genomes were obtained in concatenated form from PAMDB website http://pamdb.org. Genes and their corresponding amino acid sequences spanning gum, hrp cluster were downloaded from NCBI genbank sequences of sequenced genomes. Amino acid sequences of proteins of these clusters for Xcv and Xcc were used as query to search for homology against draft genomes of Xp, Xv and Xg. The amino acid sequences were then concatenated for each pathogenicity cluster and then aligned using CLUSTALW ignoring gaps. Neighbourjoining trees were constructed with boostrap value for 1000 replicates using MEGA4 [98]. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 2723 positions in the final dataset.

Phylogeny reconstruction

Species tree. We used a supermatrix approach as in previous work [25]. Protein sequences of six *Xanthomonas* genomes (ingroups) and the *S. maltophilia* R551-3 genome (outgroup) were clustered in 5,096 families using OrthoMCL [99]. We then selected families with one and only one representative from each of the ingroup genomes and at most one outgroup protein, resulting in 2,282 families. Their sequences were aligned using MUSCLE [100] and the resulting alignments were concatenated. Non-informative columns were removed using Gblocks [101], resulting in 792,079 positions. RAxML [102] with the PROTGAMMAWAGF model was used to build the final tree.

Prediction of effector repertoires, cloning of candidate effectors and confirmation using avrBs2 reporter gene assay

A database was created collecting all the known plant and animal pathogen effectors. Using all these known effectors as query, tblastn analysis was performed against all contigs of the draft genomes of Xv, Xg and Xp with e-value of 10^{-5} [103]. Pfam domains were searched for possible domains found in known effectors in predicted set of ORFs of draft genome sequences. Candidate effectors were classified according to the nomenclature and classification scheme for effectors in xanthomonads recently [24]. Candidate effectors showing < 45% identity at amino acid level to the known effectors were confirmed for their translocation using *avrBs2* reporter gene assay.

N-terminal 100 amino acid region along with upstream 500 bps sequence of candidate genes were PCR amplified using primers with BgIII restriction sites at the 5' ends. Following digestion with BglII, PCR amplicons were ligated with BglII-digested pBS(BglII:: avrBs262-574::HA) (courtesy of Dr. Mary Beth Mudgett, Stanford university), and later transformed into E. coli DH5 α . In-frame fusions were confirmed by DNA sequencing using F20 and R24 primers. BamHI-KpnI fragments containing the candidate gene fused to avrBs2 was then cloned into pUFR034. Resulting plasmids were then introduced into Xcv pepper race 6 (TED3 containing mutation in avrBs2) by tri-parental mating. The resulting Xcv strains were inoculated on Bs2 pepper cv. ECW 20R and kept at 28°C in growth room. After 24 hours, strong HR was indicating successful translocation of candidate effector fusions.

Cloning of pepper specificity genes in Xp

The three genes mentioned above were cloned individually and in combination in pLAFR3 vector and conjugated in Xp 91-118 $\Delta avrXv3$ mutant PM1. The PM1 transconjugants with the three individual genes and combined ones along with virulent pepper race 6 strain were infiltrated at 10⁵ CFU/ml concentration in pepper cv. ECW and leaves were sampled at every 48 hours after inoculation. The samples were plated on nutrient agar, incubated at 27°C and CFU/ml counts were enumerated. Experiment was carried out in triplicate and repeated three times.

Database submission

The draft genome sequences of *Xanthomonas vesicatoria* ATCC 35937 (Xv) have been deposited at DDBJ/EMBL/ GenBank under accession number AEQV00000000. The draft genome sequences of *Xanthomonas perforans* 91-118 (Xp) have been deposited at DDBJ/EMBL/GenBank under accession number AEQW00000000. The draft genome sequences of *Xanthomonas gardneri* ATCC 19865 (Xg) have been deposited at DDBJ/EMBL/GenBank under accession number AEQX00000000. The version described in this paper is the first version, AEQV01000000, AEQW01000000, AEQX01000000. All three draft genomes will be released upon manuscript acceptance.

Additional material

Additional file 1: Table S1: General features of the sequencing data and of the *de novo* assemblies of draft genomes of *Xv*, *Xp* and *Xg* using individual sequencing methods.

Additional file 2: Figure S2: 1a) Phylogenetic tree based on MUMi indices; 1b) Distance matrix based on MUMi indices. MUMi program was used to calculate pairwise distances between draft genomes and reference *Xanthomonas* genomes.

Additional file 3: Table S3: Whole genome comparisons using MUMmer dnadiff program. % coverage of the aligned contigs and % identities of the respective contigs against reference genomes has been shown for each draft genome.

Additional file 4: Figure S4: AvrBs2-based HR assay confirms translocation of novel effectors. Hypersensitive response reaction indicating presence of translocation signal was recorded 24 hrs after inoculation on pepper cv. ECW20R with candidate effectors *xopZ2* (a), *avrBs1* (b), *xopG* (d), *xopAM* (e), *xopAO* (f) conjugated in race 6 strain along with control race 6 strain (c). All the strains showed water-soaking on pepper cv. ECW after 48 hrs after inoculation

Additional file 5: Table S5: Evidence of the horizontal gene transfer using Alien_hunter analysis.

Additional file 6: Table S6: Genes/contigs representing T6SS in draft genomes as compared to Xcv.

Additional file 7: Table S7: Domain architecture and distribution of proteins with HD-GYP, GGDEF and/or EAL domains encoded by genomes of different *Xanthomonas* strains.

Additional file 8: Table S8: Genes unique to *Xp*, grouped in clusters. Additional file 9: Table S9: Genes common to all pepper pathogens but absent from *Xp*.

Additional file 10: Table S10: Genes unique to Xg. Genes of special interest are highlighted in red and yellow.

List of abbreviations

Xcv: Xanthomonas euvesicatoria strain 85-10; formerly, Xanthomonas campestris pv. vesicatoria strain 85-10; Xv: Xanthomonas vesicatoria strain 1111 (ATCC 35937); Xp: Xanthomonas perforans strain 91-118; Xg: Xanthomonas gardneri strain 101 (ATCC 19865); Xoo: Xanthomonas oryzae pv. Oryzae; Xcc: Xanthomonas campestris pv. Campestris; Xcm: Xanthomonas campestris pv. musacearum NCPPB4381; Xvv: Xanthomonas vasicola pv. vasculorum NCPPB702; Xac: Xanthomonas citri subsp. citri strain 306; formerly, Xanthomonas axonopodis pv. citri strain 306; Xaub: Xanthomonas fuscans subsp. aurantifolii B strain; Xauc: Xanthomonas fuscans subsp. aurantifolii C strain; Xoc: Xanthomonas oryzae pv. Oryzicola; Xca: Xanthomonas campestris pv. Armoracie; Xalb: Xanthomonas albilineans; Sm: Stenotrophomonas maltophilia.

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Authors' contributions

JBJ conceived the project. JBJ and BJS oversaw genomic sequencing. JBJ provided the strains. KK did genome assembly. NP, RPR, RK, VC, PBP, FB annotated pathogenicity clusters in the genomes. DJN and BJS helped create an effector database. NP carried out effector analysis and confirmed them experimentally. NP, BAV, RK, FFW and JBJ interpreted effector analysis. NP, RPR, RK, VC, JFP, PBP, JMD, MS, TM did analyses of different pathogenicity clusters of the three genomes and helped writing corresponding sections in the manuscript. NP oversaw the experimental validations of the pathogenicity clusters. NFA created ortholog families, and did phylogenetic analysis based on all orthologus families of the three draft genomes and the reference genomes. JCS did MUMi analysis and constructed phylogenetic tree based on that analysis. NP generated the GenBank files. DJN, BJS, FFW, RK, JBJ, BAV, JFP, and JCS helped with data analyses. NP and JBJ wrote the final manuscript. All authors approved the final manuscript.

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